



IDENTIFICATION OF FREE AMINO ACIDS IN SEVERAL CRUDE EXTRACTS OF WAX BEANS (*P. VULGARIS*) USING THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

Phaseolus vulgaris, is a common bean, which is used as edible food. In this paper an attempt have been done to identify the free amino acids in it by using chromatographic technique (TLC). It was found that various essential and non essential amino acids were present in *Phaseolus vulgaris*,. and they can be identified easily by TLC.

KEYWORDS: *Phaseolus vulgaris*, essential and non essential amino acid, Protein, Thin layer chromatography.

INTRODUCTION

Phaseolus vulgaris, the common bean,^[1] is a herbaceous annual plant grown worldwide for its edible dry seeds or green, unripe pods. Its leaf is also occasionally used as a vegetable and the straw as fodder. Its botanical classification, along with other *Phaseolus* species, is as a member of the legume family Fabaceae. Like most members of this family, common beans acquire the nitrogen they require through an association with rhizobia, which are nitrogen-fixin. The common bean has a long history of cultivation. All wild members of the species have a climbing habit,^[2,3] but many cultivars are classified either as *bush beans* or *climbing beans*, depending on their style of growth. The other major types of commercially grown beans are the runner bean (*Phaseolus coccineus*) and the broad bean (*Vicia faba*). Beans are grown on every continent except Antarctica. Worldwide, 27 million tonnes of dried and 24 million tonnes of green beans were grown in 2016.^[4] In 2016, Myanmar was the largest producer of dried beans, while China produced 79% of the world's total of green beans. Green beans may have a purple rather than green pod, which changes to green when cooked.^[4] Wax beans are *P. vulgaris* beans that have a yellow^[2] or white pod. Wax bean cultivars are commonly grown; the plants are often of the bush or dwarf form.

Essential amino acids, also known as indispensable amino acids, are amino acids that humans and other vertebrates cannot synthesize from metabolic intermediates. These amino acids must be supplied from an exogenous diet because the human body lacks the

metabolic pathways required to synthesize these amino acids.^[5,6] In nutrition, amino acids are classified as either essential or non-essential. These classifications resulted from early studies on human nutrition, which showed that specific amino acids were required for growth or nitrogen balance even when there is an adequate amount of alternative amino acids.^[7] Although variations are possible depending on the metabolic state of an individual, the general held thought is that there are nine essential amino acids, including phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine, and lysine. The mnemonic PVT TIM HaLL ("private Tim Hall") is a commonly used device to remember these amino acids as it includes the first letter of all the essential amino acids. In terms of nutrition, the nine essential amino acids are obtainable by a single complete protein. A complete protein, by definition, contains all the essential amino acids. Complete proteins usually derive from animal-based sources of nutrition, except for soy.^[8,9] The essential amino acids are also available from incomplete proteins, which are usually plant-based foods. The term "limiting amino acid" is used to describe the essential amino acid present in the lowest quantity in a food protein relative to a reference food protein like egg whites. The term "limiting amino acid" may also refer to an essential amino acid that does not meet the minimal requirements for humans.^[10]

Amino acids are primary metabolites and nutritional organic compounds that are the building blocks of proteins.^[11] The standard (proteinogenic) amino acids consist of an amine group (NH₂), a carboxylic acid (COOH), a hydrogen, and a side chain.^[12] Thin-layer

chromatography (TLC) is a simple, qualitative, sensitive, and widely used method for the separation and identification of amino acids in plant extracts. Amino acids vary in their solubility in water and organic solvents depending on the nature of their side chains; water-soluble amino acids have polar side chains.^[13]

In general, 70% aqueous ethanol is the most preferable solvent to prepare crude extracts prior to the TLC analysis of amino acids.^[14]

In this study, the amino acid profiles of seed cotyledons and seed coats from two types of legumes: black-eyed beans (*Vi-gna unguiculata*) and red kidney beans (*Phaseolus vulgaris*) were determined and compared in order to evaluate their nutritional values as major source of plant amino acids. To improve the separation and detection of free amino acids, the TLC technique was applied on several crude extracts obtained at the primary extraction stage using a range of polar and nonpolar solvents. The obtained crude extracts were:

1. Kidney bean seeds 70% ethanol (KBS 70% ethanol)
2. Kidney bean seeds 100% methanol (KBS 100% methanol)
3. Kidney bean seeds 100% acetone (KBS 100% acetone)
4. Kidney bean seed coat 70% ethanol (KBSC 70% ethanol)
5. Kidney bean seed coat 100% methanol (KBSC 100% methanol)
6. Black-eyed bean seeds 70% ethanol (BEBS 70% ethanol)
7. Black-eyed bean seeds 100% methanol (BEBS 100% methanol)
8. Black-eyed bean seeds 100% acetone (BEBS 100% acetone)
9. Black-eyed bean seed coat 70% ethanol (BEBSC 70% ethanol)
10. Black-eyed bean seed coat 100% methanol (BEBSC 100% methanol)
11. Kidney bean seeds 100% chloroform (KBS 100% chloroform)
12. Black-eyed bean seeds 100% chloroform (BEBS 100% chloroform)

MATERIALS AND METHOD

All instrument settings are those recommended in the manufacturer's manual. The instrument was calibrated using appropriate internal chemical standards (Merck Chemicals).

Preparation of Crude Extracts

Two kilograms of black-eyed beans and two kilograms of red kidney beans were soaked in distilled water for 30 and 45 min, respectively, to remove the seed coat. Then, seeds and seed coats were left to dry at room temperature. After that, the seeds and seed coats were ground to flour using a coffee grinder and defatted using a nonpolar solvent (hexane). The ground seeds and seed coats were extracted using solvent extraction methods^[15] in which

solvents with different polarities were used in Soxhlet extraction.^[16] For both the decorticated seeds and seed coats, a 500-mL amount of solvent was needed for the extraction of 100 g of flour along the extraction process. The process continued until the resulting filtrate was colorless, indicating that most of the soluble constituents have been extracted.^[17] The following selective aqueous and organic solvents were used to prepare the crude extracts; dehumidify the samples by holding them at 60°C for 6 hours. The accuracy of the digestion method was assessed by adding aliquots of metal standards, which were then analyzed as usual. Acceptable yields of >96% were obtained for the metals analyzed.

Aqueous-ethanol (70% ethanol), polarity = 1.0 for water and 0.65 for ethanol

Methanol (100%), polarity = 0.76

I. Acetone (100%), polarity = 0.36.

Chloroform (100%), polarity = 0.26

Extracts were then suction-filtered and evaporated to dryness under vacuum in a rotary evaporator (Rotavapor® R-210/R-215) at the boiling point of the solvent. The dry extracts were preserved at 4°C prior to the analysis of amino acids.^[18]

Thin-Layer Chromatography

The amino acid profiles of all the extracts were determined using thin-layer chromatography (TLC). The following fresh solutions were prepared to run the TLC:

- I. Mobile phase: *n*-butanol-acetic acid-glacial-water, mixed in a ratio of 12:3:5, respectively.^[19]
- II. Visualization reagent: ninhydrin reagent: 0.2 g/100 mL ethanol.
- III. Amino acid standards: the following amino acid standards were dissolved in water to a concentration of 2% (w/v) and used for TLC spotting: glycine, serine, leucine, cysteine, valine, aspartic acid, tryptophan, tyrosine, threonine, histidine, proline, glutamic acid, cystine, arginine, alanine, glutamine, isoleucine, asparagine, methionine, phenylalanine, hydroxyproline, and lysine.

Using capillary tubes, small spots of amino acid standards and bean extract solutions were applied 2 cm above one edge of silica-coated TLC plates (Whatman silica gel 60 A, 20 × 20 cm, 250 µm thickness with fluorescent indicator) with a 1-cm space between the spots. The spots were then labeled with a pencil and left to dry at room temperature before being located vertically in a TLC chamber containing the developing solution (the mobile phase). The bottom edge of the plate beneath the initial sample spots was immersed in the developing solution, and the TLC chamber was sealed. Then, the plates were left to stand for approximately 3 h. The highest point reached by the mobile phase was marked. When the plates were completely dry, they were sprayed with the ninhydrin reagent and left at 80–100°C for 5 min to allow the visualizing reagent to react and stain the spots. The distances from the starting point to

the spot centers were measured, and the retardation factor (R_f) value for each standard and unknown spot was calculated (R_f = the distance traveled by the spot / the distance traveled by the mobile phase front).

The following symbols were used to describe the results of the detected amino acids:

(+) observed, (++) bright, and (□) faint.^[20]

RESULTS AND DISCUSSION

The organic solvents used to produce the crude extracts varied in their abilities to extract amino acids; no amino acids were detected in chloroform fractions. Sixteen amino acids were extracted by different polar solvents. The calculated R_f value for each standard is shown in **Table 1**. These R_f values were used as markers to identify the amino acids in each extract (**Table 2**).

The results obtained from the TLC (Tables 1 and 2) indicate that the amino acid profile of the kidney beans and the black-eyed beans varied with different amino acids detected in different crude extracts. Some amino acids such as Ala, Asp, Glu, Pro, Leu, and Val were poorly or not detected in the crude extracts prepared with 70% aqueous ethanol. All of the essential amino acids with the exception of Met were found in the kidney-bean extracts, while Arg, Asn, Met, Gly, and His were absent from the black-eyed bean extracts. Most of the amino acids were detected in the seed cotyledon fractions, with only a few detected in the seed coat

extracts. More amino acids were detected in the black-eyed bean seed coat than the kidney bean seed coat; the 70% ethanol fraction of black-eyed bean seed was found to contain Ala, Cys, Glu, Tyr, and Val, and the 100% methanol fraction contains Ala, Glu, Tyr, and Val. Only one fraction of the kidney bean seed coat was found to have amino acids: the 70% ethanol extract which was found to contain Cys, Phe, and Thr. Amino acids have different functions in different legume seeds; Cys is a sulfur-containing amino acid that plays an important role in plant defense mechanisms against predators and is found to be toxic to cowpea beetles *Callosobruchus maculatus*.^[21] This may explain why seed coats help protect legumes against stored seed pests and minimize losses of stored products. Moreover, both types of beans seem to lack the following amino acids: Hyp, Ser, Gln, L-Cys, Try, and Met which reduces the nutritional value of these beans. Combining beans with other legumes or grains is recommended to provide a meal that is rich in all essential amino acids.

Table 1: Essential and nonessential amino acid standards; abbreviations, polarity, and R_f values obtained from the TLC on silica plates, eluted with *n*-butanol–acetic acid–water mixture 12:3:5 (by volume), respectively, and visualized by ninhydrin stain.

Amino acids	Abbreviations	Side-chain polarity	R_f
Histidine	His	Polar	0.162
Isoleucine	Ile	Nonpolar	0.515
Leucine	Leu	Nonpolar	0.562
Lysine	Lys	Polar	0.131
Methionine	Met	Nonpolar	0.462
Phenylalanine	Phe	Nonpolar	0.615
Threonine	Thr	Polar	0.262
Tryptophan	Try	Nonpolar	0.615
Valine	Val	Nonpolar	0.439
Alanine	Ala	Nonpolar	0.277
Arginine	Arg	Polar	0.162
Asparagine	Asn	Polar	0.215
Aspartic acid	Asp	Polar	0.24
Cysteine	Cys	Polar	0.362
L-Cystine	L-Cys	Polar	0.139
Glutamic acid	Glu	Polar	0.277
Glutamine	Gln	Polar	0.223
Glycine	Gly	Nonpolar	0.246
Proline	Pro	Nonpolar	0.262
Serine	Ser	Polar	0.254
Tyrosine	Tyr	Polar	0.539
Hydroxyproline	Hyp	Polar	0.426

Table 2: Amino acids detected in extracts of seed cotyledon and seed coat of kidney beans and black-eyed beans identified by their *R* values refer- ring to the amino acid standards' *R* values.

Extracts	KBS 70%	KBS 100%	KBS 100%	KBSC 70%	KBSC 100%	BEBS 70%	BEBS 100%	BEBS 100%	BEBS 70%	BEBS 100%
Amino acids	ethanol	methanol	acetone	ethanol	methanol	ethanol	methanol	acetone	ethanol	methanol
Ala		++	+				+	±	±	+
Arg	+		+							
Asn	±									
Asp			±			+				
Cys				+					+	
Glu			+					±	±	+
Gly	+	+								
Pro			+				+			
Tyr	±	+	+				++	++	++	+
His	±	+	±							
Ile	+	+	+			+				
Leu		++				+	±			
Lys	+	+	±			+	±			
Phe		+		+				±		
Thr	+	±		+			+			
Val	+	++	+			+	+	±	+	+

CONCLUSION

TLC is an efficient method for the analysis of amino acids from plant materials. Amino acids vary in their polarities according to their side chains. Producing several crude extracts is a vital first step for further separation steps. Using several solvents, with different polarities in crude extracts preparation, improves the efficacy of the TLC technique, enhances separation, and simplifies the identification of polar and nonpolar amino acids.

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